Partial Mitochondrial DNA Barcode of Rafflesia Mira Fernando & Ong, 2005 (Syn. Rafflesia Magnifica Madulid, Tandang, Agoo, 2005) Using MatR with Phylogenetic Analysis of Selected Rafflesia Species in the World

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Abstract— *In this paper, partial DNA barcode of R.mira syn. R.magnifica a critically endangered holoparasitic plant from Maragusan Compostela Valley Province was provided using mitochondrial gene sequence matR. Partial matR sequences generated from 3 replicates of R.mira (syn.R.magnifica) showed very close genetic variability between Philippine endemic species R. speciosa and R. manillana as shown in phylogenetic tree using neighbor-joining with bootstrap support and confirmed by pair-wise distance analysis.*

Keywords—DNA, matR, Rafflesia mira, R. magnifica, Maragusan, Compostela Valley.

I. INTRODUCTION

Rafflesia, a genus of approximately 20 plus species worldwide notable in having one of the largest flowers that measures approximately 1 meter in diameter and weigh 7 kg (Nais, 2001). This plant group is notable for having a smell of decomposing flesh that attracts carrion flies (Beaman, 1988). As endoparasitic plants embedded within their host plants, *Rafflesia* and other parasitic relatives such as *Rhizanthes* and *Sapria* are often hardly believed to be plant-like because of lacking leaves, stems, roots, and emerge only for sexual reproduction when they produce flowers (Nais, 2001).

Rafflesia mira (Fernando & Ong, 2005) syn. *R. magnifica* (Madulid et al., 2005) is the fourth *Rafflesia* species described in the Philippines in 2005 (Madulid et al., 2005). Second from the largest which is *R. schadenbergiana*, this species is found only at Mt. Candalaga Range in Maragusan, Compostela Valley Province Philippines. Madulid et al. (2005) suggested that strong conservation efforts must be conducted for *R.mira* (syn. *R. magnifica*) since it is not found within protected landscape unlike all other *Rafflesias* described in the Philippines. Due to its very limited habitat range and prone to various anthropogenic disturbances with decreasing population trend (Madulid, et al. 2005), this species is labeled as critically endangered (IUCN, 2015-4 v.3.1).

Based on molecular evidence, it is believed that *Rafflesia* and the rest of the members of the order Rafflesiales came from polyphyletic origin as shown by molecular studies (Nickrent et al., 2004). More recently, *Rafflesia* are placed amongst Malpighiales based on molecular phylogenetic studies (Barkman, et al., 2004; Nickrent et al., 2004). Experimental trials of Lin and Ritland (199) showed that spectrophotometric measurements of isolated DNA show that corollas yield much more DNA than other parts of the plant. In this paper, DNA isolation protocol had generated good quality DNA isolates. Moreover, it is not the intention of this paper to resolve that the two (*R.mira* and *R.magnifica*) are the same or different species, but only to provide DNA barcode useful in further genetic studies and species conservation. For the first time, DNA barcode using mitochondrial gene sequence *matR* of *R. mira* (syn. *R.magnifica*) was provided with phylogenetic analysis of selected *Rafflesia* species utilizing *MatR* sequence.

II. MATERIALS AND METHODS

Plant tissue collection: Permit from the Office of the Mayor thru the Department of Environment and Natural Resources Office (DENR)- Municipal Environment and Natural Resources Office (MENRO) and the Tourism

Office of the Municipality of Maragusan was secured prior to the collection of tissue samples. Identification of specimen was confirmed using published materials of Fernando & Ong (2005) and Madulid et al. (2005. With the use of sterile lancet, small portion of tissues from the plant was isolated. Tissues were placed in a paper coin envelope containing silica gels used to absorb extra moisture from the tissues. Tissue samples were brought to the Biofrezzer of Natural Science Research Center (NSRC), Central Mindanao University, Musuan Bukidnon.

DNA Extraction & Gel electrophoresis: Each tissue samples were weighed using electronic balance, placed in a sterile mortar and pestle added with liquid nitrogen and grinded into fine powder. Using sterile scoop, fine tissue powder were transferred into microtubes carefully added with 800ul extraction buffer pre-heated at 50 celcius. 1ul *B*-mercaptoethanol was added to denature irreversibly *RNAses* by reducing disulfide bonds and destroying the native conformation required for enzyme functionality (Gong & Lim, 2009). Samples were mixed using vortex and incubated for 25 minutes at 60^{o}C. They were then added with 500ul SEVAG (24 chloroform: 1 isoamyl alcohol) and mixed for 20 minutes. Samples were then centrifuge for 15 minutes at 1500g. Aqueous layer were transferred to a fresh tube and added with equal volume of Isopropanol slowly added with 0.5 (half) volume of 5M NaCl (-70^{o} C for 1 hour), centrifuge at 11000 RPM for 10 minutes and gradually decant aqueous layer and then air dry (or using laminar flow). Tubes were washed with 70% ethanol and centrifuge at 11000 RPM for 4 minutes. Samples were then diluted with 50ul molecular grade sterile water and added with 1 ul of 1mg/mL RNAse and incubated for 30 minutes at 37^{o}C. 2 volumes of ethanol and 0.1 volume of 3M NaAC, pH 5.0 were added and centrifuge for 10 minutes at 11000 RPM at 40^{o}C. Washed again with 70% ethanol, gradually removed the ETOH, and air dried for 5 minutes. Samples were then re-suspended in TE buffer 50µL TE (10mM TrisHCl pH 8.0, 0.1mM EDTA pH 8.0) and treated with 1µL of RNAaseA (10mg mL-1) at 50°C for 10 minutes. Using 2:5 ul ratio of loading dye and samples were loaded. Samples were carefully loaded to the loading wells and run the gel electrophoresis for 30 minutes. The gel was analyzed using Biorad gel imager.

DNA amplification: DNA amplification was performed using *MatR* 5' forward GTTTTCACACCATCGACCGACATCG and *MatR* 3' reverse (CGCGGCACCTGTAGTAGGACAGAGGA (Anderberg, et al. 2002; Barkmann, et al. 2004) under the following thermal cycling conditions: 94^{0}C for 2.5 min followed by 35 cycles of 94^{0} C for 0.5 min, 54^{0} C for 1 min, and 72^{0} C for 1.5 min, and then by a final extension at 72^{0} C for 3 min. Each PCR reaction included 5 □ l of 10□ Rxn buffer (Invitrogen), 2.5 mM $MgCl_2$, 400 □ M dNTPs, 0.2 □ M of each primer, 0.5 unit of Platinum *Taq*DNA Polymerase (Invitrogen), ~0.5 ng of total DNA, and enough H_2O to bring the volumes up to 50 □ l.

PCR protocol & Sequencing: PCR cocktail was prepared by mixing 6.4ul sterile water, 1.0 Buffer ($MgCl_2$), 0.8 dNTP, 0.2 DNA primers (forward & reverse), 0.3 MgCl2, 0.04 *Taq* pol. All preparations were dissolved in 9 ul H_2O + 1ul DNA sample. Dilution was made (if necessary) by 1:100 molecular grade water or 1:500 molecular grade water depending on the thickness of DNA sample in gel. PCR runs under the following thermal cycling conditions: Stage 1, 1 cycle at 94^{o}C in 4 minutes; Stage 2, 5 cycles at 94^{o}C, 55^{o}C, and 72^{o}C in 30 seconds, 1 minute, and 1 minute respectively; Stage 3, 30 cycles at 94^{o}C, 54^{o}C, 72^{o}C in 30 seconds, 1 minute, and 1 minute respectively; And Stage 4, 1 cycle at 72^{o}C in 10 minutes. DNA products were confirmed after loading back the samples to gel electrophoresis. DNA products were sent to Macrogen, South Korea for sequencing.

Bioediting and construction of Phylogenetic tree: DNA sequence was first confirmed that it is from genus *Rafflesia* using NCBI nucleotide BLAST, a U.S. government-funded national resource for molecular biology information (www.**ncbi**.nlm.nih.gov/). Bioediting and construction of phylogenetic tree were conducted using Bioedit software (http://en.freedownloadmanager.org/Windows-PC/BioEdit-FREE.html) and MEGA 6.0 (http://www.megasoftware.net/) respectively.

III. RESULTS AND DISCUSSION

The taxonomic classification of Rafflesia in the study is as follows: Domain Eukaryota, Kingdom Plantae, Division Tracheophyta, Order Malphigiales, Family Rafflesiaceae, Genus *Rafflesia*, Species *mira* (syn. *R.magnifica*). The recent publication of Galindong, Ong, and Fernando, 2016 added a new species of Rafflesia from Luzon Island, Philippines making the country as one of the center of *Rafflesia* diversity with at least 13 species described (Teschemacher 1842, Blanco 1845, Hieronymus 1885, Barcelona and Fernando 2002, Fernando and Ong 2005, Barcelona et al. 2006, 2008a, 2008b, 2009a, 2009b, 2011, 2014, Galang and Madulid, 2006, Balete et al. 2010, Pelser et al. 2013). *Rafflesia mira* (syn. *R.magnifica*) showcased in this paper is listed as critically endangered species due to very small habitat range, and the habitat itself

is not covered by Protected Area making the species very prone to extinction (Madulid et al. 2005).

Percent sequence identity: The present DNA sequence is 97% related to two Philippine endemic species, *R. manillana* syn *R.cumingii*, Brown, (Barcelona et al. 2009) and *R. speciosa* from Antique Panay Island with total scores of 2316 and 2305 respectively (https://blast.ncbi.nlm.nih.gov/Blast.cgi). It is also closely related to Malaysian species, *R.tengku-adlinii*, *R.keithii* at 97% identical with 2327 and 2322 total scores. This sequence is also identical at 97% to other Malaysian species, *R.pricei*, *R.cantleyi*, *R.tuan-mudae*, *R.hasseltii*, *R. gadutensis*, *R. kerrii* and *R. patma*. It is 96% identical to *R.arnoldii* and *R.zollingeriana*.

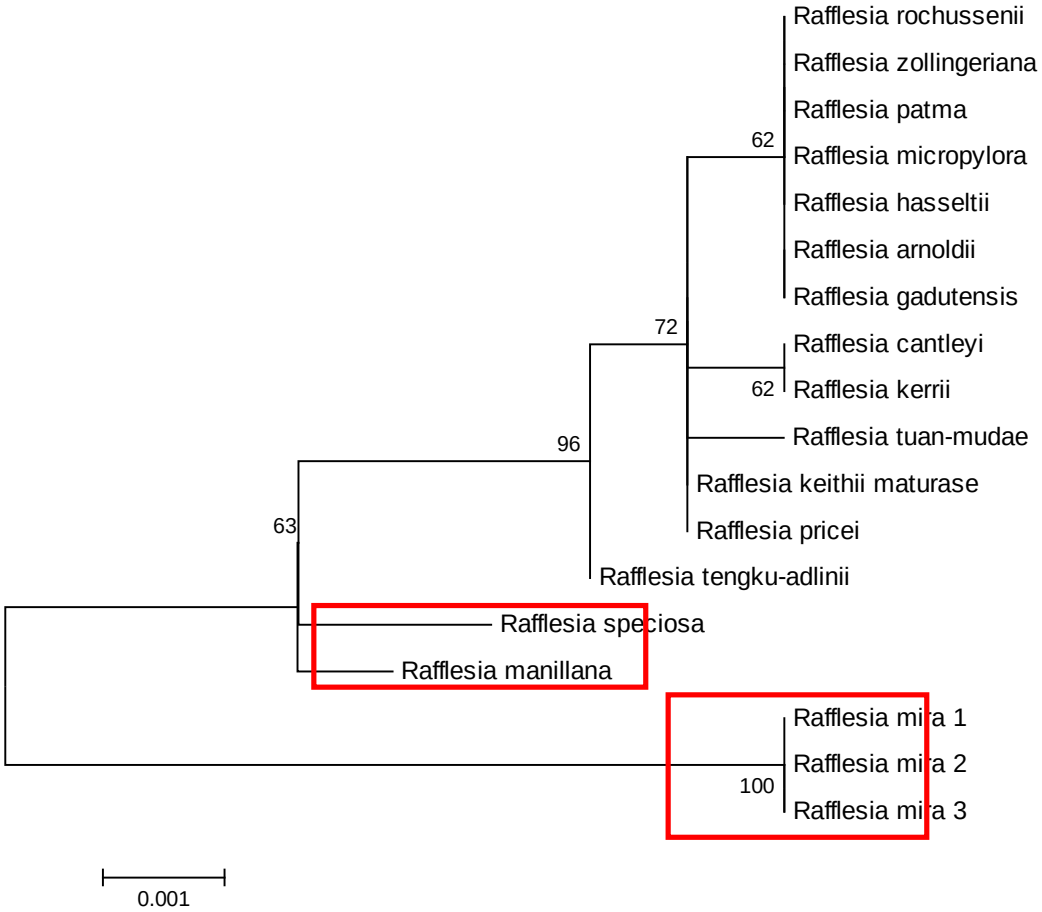

Fig.1: Phylogenetic tree from partial matR sequences of 18 individuals of sixteen Rafflesia species generated using the Neighbour-Joining (NJ) method with Maximum Composite Likelihood as the measure of evolutionary distance. Bootstrap values (%) are shown on each branch.

Using Neighbor-Joining method (Saito & Nei, 1987) the partial evolutionary history was inferred. The sum of branch length = 0.01673581 from optimal tree was shown. The phylogenetic tree was inferred from drawing to scale the phylogenetic tree with branch lengths in the same units as those of the evolutionary distance. Maixmum Composite Likelihood Method was used to compute the evolutionary distances (Tamura, Nei, & Kumar, 2004) with 18 nucleotide sequences involved in the analysis. The 1st, 2nd, 3rd, and Non-coding sequence positions were included. We also eliminated the positions containing gaps and missing data. A total of 1261 positions in the final dataset and were analyzed using MEGA6 software (Tamura et al. 2013).

MatR sequences revealed close relationship between *R. mira* and its co-Philippine endemic species *R. manillana* and *R. speciosa* as shown in the phylogenetic tree (Fig. 1). Not surprisingly, a close relationship was observed between species located in the same geographic location. Results conform to the study of Webb (2000) in Borneo that tree species occupying a 0.16-hectare plots in a rainforest in

Borneo seemed to be more closely related to one another than expected by chance assembly from the regional species pool of 324 tree species. There is a possibility that *R.mira* and other Philippine endemic *R. manillana* and *R. speciosa* descended from a common ancestor. Genetic variability of Philippine species could be accounted to changes in the topography, gradient, and other environmental factors. Distance between population and other discrete geographic barriers would play a role (Reis et al. 2015), as seed dispersal becomes limited for holoparasitic plants like *Rafflesia*. In addition, geographic barrier between nearby populations may also suffer from ecological isolation caused by habitat heterogeneity leading to divergence by local adaptation and drift.

Elevation gradients may also influence genetic structure and diversity (Reis, et al. 2015; Mayr, 1963) particularly for holoparasitic which are restricted to host plants. In addition, a significant positive correlation between genetic and geographic distance was observed with the genetic structure for an Atlantic rain forest tree (*Bathysa australis*) in the Serra do Mar mountain range, Southeast of Brazil (Reis et al. 2015). Generally, populations at intermediate altitudes have greater diversity compared to lower and higher altitudes. Primarily because of the optimal conditions in the mid-range while the peripheral populations experiencing suboptimal situations (Ohsawa & Ide, 2008).

R. speciosa was discovered in lowland secondary dipterocarp of Panay Island at 560-680 masl (Barcelona & Ong, 2002) and R.manillana was discovered in the lowland forest of Western, Samar at approximately 1000 masl (Madulid & Agoo, 2008) and *R. mira* (syn. *R.magnifica*) was found only at the mid-montane forest at around 900 masl with semi-open habitats of Compostela Valley Province (Fernando & Ong, 2005; Madulid, Tandang, Agoo, 2005). As observed that the three closely related species have almost the same habitat types and elevation which could also explain their very close genetic variability, although sub-populations of *R.manillana* in Mt. Makiling was recorded from 436-834 meters elevation (Yahya et al. 2010).

Table 1: Pair-wise distances or estimates of evolutionary divergence between sequences of sixteen species of Rafflesia.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
*R.mira*_R1	----------																
*R.mira*_R2	0.0000																
*R.mira*_R3	0.0000	0.0000															
R.manillana	0.0096	0.0096	0.0096														
R.speciosa	0.0104	0.0104	0.0104	0.0024													
R.arnoldii	0.0128	0.0128	0.0128	0.0048	0.0056												
R.cantleyi	0.0128	0.0128	0.0128	0.0048	0.0056	0.0016											
R.gadutensis	0.0128	0.0128	0.0128	0.0048	0.0056	0.0000	0.0016										
R.hasseltii	0.0128	0.0128	0.0128	0.0048	0.0056	0.0000	0.0016	0.0000									
R.keithii	0.0120	0.0120	0.0120	0.0040	0.0048	0.0008	0.0008	0.0008	0.0008								
R.kerrii	0.0128	0.0128	0.0128	0.0048	0.0056	0.0016	0.0000	0.0016	0.0016	0.0008							
R.micropylora	0.0128	0.0128	0.0128	0.0048	0.0056	0.0000	0.0016	0.0000	0.0000	0.0008	0.0016						

R.patma	0.0128	0.0128	0.0128	0.0048	0.0056	0.0000	0.0016	0.0000	0.0000	0.0008	0.0016	0.0000					
R.pricei	0.0120	0.0120	0.0120	0.0040	0.0048	0.0008	0.0008	0.0008	0.0008	0.0000	0.0008	0.0008	0.0008				
R.rochusseni i	0.0128	0.0128	0.0128	0.0048	0.0056	0.0000	0.0016	0.0000	0.0000	0.0008	0.0016	0.0000	0.0000	0.0008			
R.tengku-adlinii	0.0112	0.0112	0.0112	0.0032	0.0040	0.0016	0.0016	0.0016	0.0016	0.0008	0.0016	0.0016	0.0016	0.0008	0.0016		
R.tuan-mudae	0.0128	0.0128	0.0128	0.0048	0.0056	0.0016	0.0016	0.0016	0.0016	0.0008	0.0016	0.0016	0.0016	0.0008	0.0016	0.0016	
R.zollingeriana	0.0128	0.0128	0.0128	0.0048	0.0056	0.0000	0.0016	0.0000	0.0000	0.0008	0.0016	0.0000	0.0000	0.0008	0.0000	0.0016	0.0016

Table 1 shows the pair-wise distances between different species of Rafflesia (range: d=0.0000 – 0.0128). The very low genetic divergence estimates suggests very close relationships between Rafflesia species particularly for Philippine endemic species *R. mira* (syn. *R.magnifica*), *R.speciosa*, and *R. manillana* (syn. *R.cumingii*) (range: d=0.000-0.0104). There is a slight change of genetic diversity when pair-wise distances between Philippine endemic species and other oriental species from Malaysia and Borneo (range: 0.0104-0.0128). This genetic divergence estimates is consistent with phylogenetic tree analysis where Philippine endemic species *R. mira* (syn. *R.magnifica*), *R.speciosa*, and *R. manillana* (syn. *R.cumingii*) are more likely closely branched compared to species from Malaysia and Borneo.

Partial DNA barcode using *matR*

>*R. mira* (syn. *R. magnifica***)**

ATAAAGTCTTTTCCGCCAGACGACTCGTAGGAGGT
GAAATGCCCCGGACTCCGCCCACACAGTGTACTAC
TATCGGCCCTACCAGGCAACATATACCTACACAAG
TTCGATCAGGGAATAGGGGGGGTTCCGCCCTAAGT
ACGAAATGACGATTGTTCAGAGAATAAGATCGTTC
ATGACAGGTCGTATTGATGACCAAGAACAGGATG
GAGAAGAAGCAAGCTTCCACGCTCCCCAAGGCAA
CATAGCCCTTTTTTTTTGGGATGGTAATCCAACGCA
AAGCGGCCTTTCCTTCCCTTTTTTCGTCGTGGCACA
CCCCCCGGAAAACGCCCAGGGGGGACCAGAAAAA
GCCTTCGGCACCGGCACCGGCACTTGCCGCCTTTA
TGAACAAGCCCTCGAGCCTCCTTTGCGCCGCCTTA
CTCATGGAATCCGTGACCCTTAAGGATCGCGCCGA
ATTATATGGTCGAGAACGCTGGGCCATGAGAGACC
TTATTCAGTCTTGCAAAAGAAAGGGCCTGCTGATA
GAGCTGGGCGGGGAGGCGAGACTAGTTCTCAGGT
CAGATAGACGCCTGGCCCGTAAGCTGGCTCCCTTA
AAAACCCATGACTTCATCCATCTTTCTTACGCGCG
ATATGCCAACGACTTACTACTGGGAATCGTAGGTG
CCTCCGAGCTTCTCTACGAAATACAAAAACGTATC
GCCCAATTCCTACAATCCGGCCTGACAGGCTCGGC
AGGATCAACAACAATAGCTGCACGGAGTACGGTA
GAATTCCTCGGTACGGTCATTCGGGAAGTTCCTAC
GACTATACAATTATTTCGAGAGCTGGAGAAGCGTA
TACGGGTAAAGCACCGTATCCATCTAACTGCTTGC
CACCTACGCTCCGCCATCCATTCAAAGTTTAGGAA
CCTAGGTAAGAGTATCCCGATCAAACAGCTTACGA
AGGAAATGAGCGAAAAAGGGAGTCTACTGGGACG
GGGTACCGGAAAACTCTTGGAACAACTGGGAGTA
AGAAAGTCCCCAAGCGAAGCGTATTATGGGGGGA
CATTCCAGCACATCCGGCAAGGATCAAGGGGGGA
TCTCGTTTGTTGCATAGCTCAGGTCGGAGCAAGGC
CTCTCGGACGTTCAACAGGCAGTCTCACGATCGGG
CATGAGTGTCCGGTTGTATACTCCCGCGGGTTTGA
AGGCGGCGGGGGAAGGAGGAGGAGCGGGATCTAT
CAGCAGAGAATTCCCCATCAATATAGAGGCGCCTA
TCAAAAAGATACTCCGAATGCTTCAGGATCGAGGT
TTCATTAGCCGAATAAGACCCTGGCCAATCCACGT
GGCCTGCTTGACGAGCGTAAGCGACGGAGACATC
GTAAATGTCCGCGGCATCGCCGATTCGG

IV. CONCLUSION

Revised DNA isolation protocol works best with *matR* primer and generated large amount of DNA products. Partial *matR* sequences generated from 3 replicates of *R.mira* (syn.*R.magnifica*) showed very close genetic variability between Philippine endemic species *R. speciosa* and *R. manillana* as shown in phylogenetic tree using

neighbor-joining with bootstrap support and confirmed by pair-wise distance analysis.

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